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REVIEW

Respiratory syncytial virus subunit vaccines based on the viral envelope glycoproteins intended for pregnant women and the elderly

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ABSTRACT

Introduction: Respiratory syncytial virus (RSV) causes high morbidity and mortality rates among infants, young children, and the elderly worldwide. Unfortunately, a safe and effective vaccine is still unavailable. In 1966, a formalin-inactivated RSV vaccine failed and resulted in the death of two young children. This failure shifted research toward the development of subunit-based vaccines for pregnant women (to passively vaccinate infants) and the elderly. Among these subunit-based vaccines, the viral envelope glycoproteins show great potential as antigens.

Areas covered: In this review, progress in the development of safe and effective subunit RSV vaccines based on the viral envelope glycoproteins and intended for pregnant women and the elderly, are reviewed and discussed. Studies published in the period 2012–2018 were included.

Expert opinion: Researchers are close to bringing safe and effective subunit-based RSV vaccines to the market using the viral envelope glycoproteins as antigens. However, it remains a major challenge to elicit protective immunity, with a formulation that has sufficient (storage) stability. These issues may be overcome by using the RSV fusion protein in its pre-fusion conformation, and by formulating this protein as a dry powder. It may further be convenient to administer this powder via the pulmonary route.

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1. Introduction

Human respiratory syncytial virus (RSV) is the most important cause of severe lower respiratory tract infections among infants and young children worldwide [1–4]. It has been estimated that, in 2015, globally 33.1 million children under 5 years of age suffered from RSV-associated acute lower respiratory infection (RSV-ALRI). Of these children, 3.2 million required hospitalization. In that same year, approximately 118,200 children deceased from RSV-ALRI [5]. RSV is also an important cause of severe morbidity and mortality among the elderly (≥65 years) [6].

The virus is transmitted via aerosols and through contact with contaminated surfaces [7]. After an initial RSV infection there is usually no long-lasting immunological memory against RSV. As a result, reinfections occur frequently [8]. Hall et al. [9] demonstrated this by challenging adults with RSV after a natural infection. Almost 50% of them were reinfected within 2 months. In the study period of 26 months, 73% were infected at least twice, and 47% were even infected three times or more. However, two subsequent infections within a short period of time tended to improve the immunological memory. Higher serum antibody levels before RSV challenge correlated with protection from infection, but the rate of reinfection was still 25% among subjects with the highest antibody titers.

Despite the significant global health burden and over 50 years of research, no vaccine against RSV is available on the market yet. Currently, palivizumab is the only approved therapeutic agent against RSV infection [10]. This monoclonal antibody binds to the fusion (F) protein of the virus [11,12]. Palivizumab is only used prophylactically with infants who are at high risk for hospitalization due to RSV infection and it needs to be administered monthly for a longer period [13]. The high costs of multiple dosing with palivizumab restricts its use, especially in low- and middle-income countries [12]. Moreover, repeated injections are a burden for the patient.

Ribavirin, a nucleoside analog, is also used for the treatment of RSV infection in high-risk patients [14]. Usually, it is administered pulmonary in an aerosolized form. Treatment with ribavirin has limitations: it is very costly, difficult to administer, and has limited clinical efficacy [12,15].

In view of the current drawbacks in RSV prophylaxis and therapy, the development of a safe and effective RSV vaccine is urgently needed. In a trial in 1966, formalin-inactivated virus (FI-RSV) was used as a vaccine. This vaccine candidate was investigated in infants and young children and yielded moderate serum antibody levels, but appeared to be unable to protect against RSV infection [16,17]. By contrast, vaccinated infants even developed more serious lower respiratory tract disease upon natural RSV infection than did infants who had not received the FI-RSV vaccine. This phenomenon is known as

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Article highlights

- The highly conserved fusion protein is a promising antigen for the development of subunit RSV vaccines for pregnant women and the elderly.
- Subunit-based RSV vaccines using the viral envelope glycoproteins as antigens elicit high virus-neutralizing antibody titers in animal models and in human trials.
- Three recent clinical studies with RSV vaccine candidates using post-fusion protein or a nanoparticle-based vaccine candidate with a morphology consistent to that of post-fusion failed, suggesting the importance of pre-F-specific antigenic sites (e.g. sites Ø and VIII) and the pre-F morphology in eliciting a protective immune response.
- A protective immune response as therapeutic outcome and the (storage) stability of the vaccine remain challenges in the development of subunit-based RSV vaccines.
- The several disadvantages of vaccine candidates described in this review may be overcome by using a dry powder pre-fusion vaccine suitable for pulmonary administration.

vaccine-enhanced respiratory disease [18]. Two of the thirty-one vaccinated children died during the trial [17]. The failure of the FI-RSV vaccine hampered research toward new RSV vaccines for many years after that study.

In recent years, however, extensive research was done on the development of an RSV vaccine and researchers are getting closer to bringing a safe and effective vaccine to the market. In December 2015, 60 different RSV vaccine candidates were in preclinical or clinical development [12]. Many different approaches have been followed in the development of an effective RSV vaccine (e.g. live-attenuated, whole inactivated, subunit-based vaccines), as different target groups for RSV vaccination require different vaccine formulations [19–21]. The different target groups for RSV vaccination are: infants (≤6 months of age), young children (6–24 months of age), pregnant women (to passively vaccinate neonates), and the elderly (≥65 years of age) [19–21]. The target group with the highest priority is infants [20]. However, since the failure of the FI-RSV vaccine, vaccinating this specific target group encounters resistance and remains elusive. On the other hand, a growing interest is seen for vaccination of pregnant women. The goal of vaccinating this target group is to induce high titers of neutralizing antibodies which are passively transferred to the fetus and thus may protect infants during the crucial first months of life [19,20,22]. According to Anderson et al. [20], subunit-based vaccines are considered for this purpose, as live RSV vaccines have not been sufficiently immunogenic in adults.

For the same reason, subunit-based vaccines are also considered for another important target group, being the elderly [20]. A major challenge of this target group is immunosenescence, which may result in an unbalanced immune response [19]. Therefore, it is likely that a subunit-based vaccine for the elderly will have to be co-formulated with adjuvants to recall pre-existing immunity and to elicit a balanced immune response [21]. For both target groups, pregnant women and the elderly, vaccine-enhanced respiratory disease is considered not to be a concern, since these target groups have been infected with a live virus multiple times [20,23]. In conclusion, subunit-based RSV vaccines show great promise for different target groups.

Several antigens can potentially be used for the development of subunit-based RSV vaccines. However, from recent literature it is clear that the F protein is considered as the most promising antigen. This is also supported by the fact that virus-neutralizing (VN) antibodies are targeted at this glycoprotein upon RSV infection [24].

This review focuses on the viral envelope glycoproteins, and in particular the F protein (in both the pre-fusion (pre-F) and the post-fusion (post-F) conformation), as antigens. The structure of RSV and the viral envelope glycoproteins are first discussed, after which recent advances in the development of subunit-based RSV vaccines, using the viral envelope glycoproteins as antigens, are reviewed. For the latter part, only studies published in the period 2012–2018 were included. The review was conducted using PubMed and the following search terms were used: respiratory syncytial virus, vaccine, attachment protein, fusion protein, virosomes, structure, hospitalization, and treatment.

2. The structure of RSV

RSV is an enveloped, negative-sense, single-stranded RNA virus, which belongs to the genus *Orthopneumovirus* of the family *Pneumoviridae* [25,26]. This family also includes human metapneumovirus, which after RSV represents the second most common cause of lower respiratory tract infections among young children [27]. Two different subtypes of RSV can be distinguished, namely RSV A and RSV B [28]. The structure of the virus is shown in Figure 1 [29]. The virus consists of a lipid envelope containing a nucleocapsid, and is pleomorphic with different sizes of spherical particles and filamentous particles [30]. The RSV genome contains approximately 15,200 nucleotides, encoding 11 different proteins [31]. The transmembrane surface glycoproteins protrude from the outside of the lipid bilayer and are 11–20 nm in size [29]. The RSV genome, proteins, and their functions are shown in Figure 2 [29].

NS1 and NS2 are non-structural proteins, which are not packaged inside RSV virions, but only detected in RSV-infected cells [32]. They mainly inhibit the production of interferon (IFN), specifically IFN-α and IFN-β, by the host cell [33]. The nucleocapsid (N) protein offers protection for the viral RNA. N and phosphoprotein (P) are essential for transcriptional activity [29]. The matrix (M) protein is important for virus assembly, as it connects the nucleocapsid with the envelope proteins [29,34]. The M2 gene encodes two proteins, M2-1 and M2-2. M2-1 functions as an elongation factor in transcription and M2-2 is involved in regulation of viral RNA transcription and replication [35,36]. The long gene L encodes the RNA polymerase of the virus [27,29], which replicates the viral RNA and is also involved in polyadenylation, capping, and methylation [29,37].

Three different proteins are present on the outer surface of the virus membrane: the F protein, the G protein, and the small hydrophobic (SH) protein [24]. The F and G proteins are the viral envelope glycoproteins, which will be discussed in more detail in the next section. The SH protein is a small protein that is found in infected cells and on the membrane of the virus [24]. The SH protein forms a pentameric ion channel complex. Its biological function is still unknown [38].

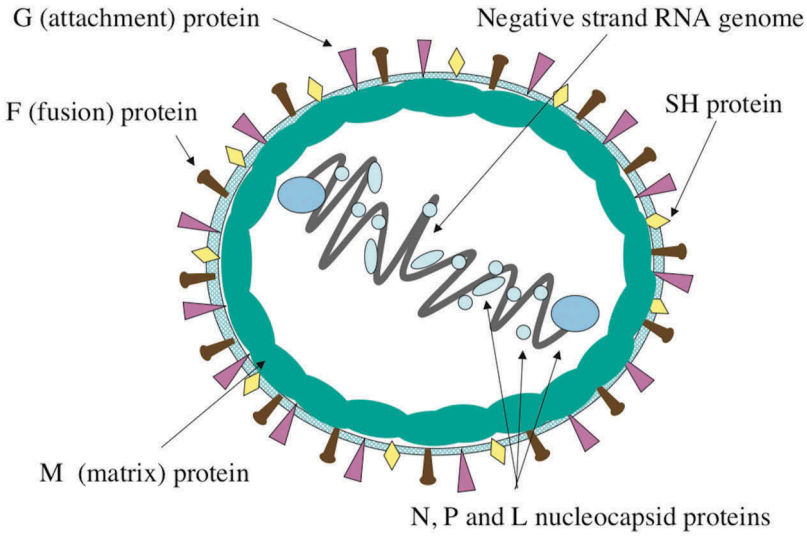

Figure 1. Structure of RSV. The nucleocapsid is a symmetrical helix and is surrounded by a lipid envelope. The surface proteins F, G, and SH protrude from the outside of the lipid bilayer. Reproduced with permission from [29].

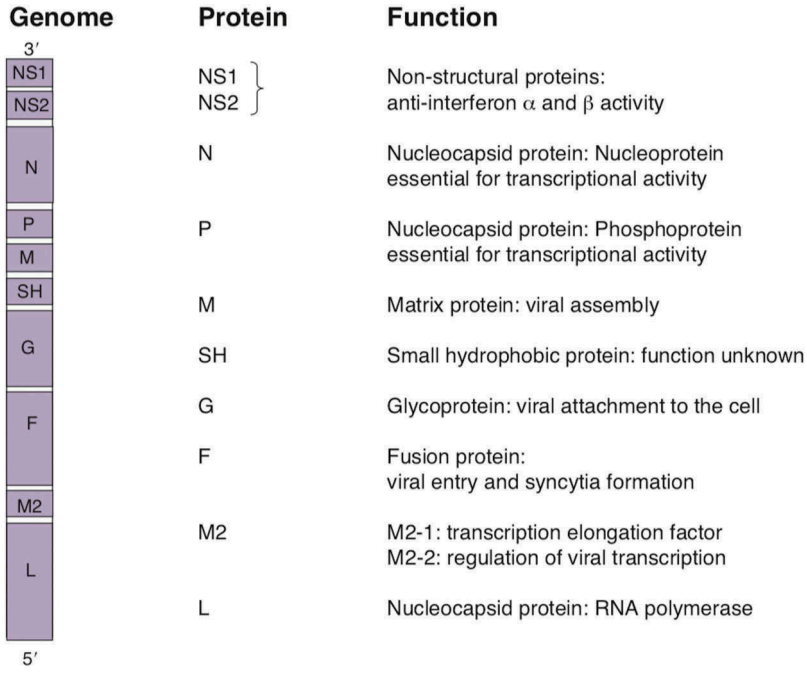

Figure 2. The RSV genome, proteins, and their functions. The genome contains 15,200 nucleotides, encoding 11 different proteins. Reproduced with permission from [29].

3. The viral envelope glycoproteins

This section is devoted to the viral envelope glycoproteins. The viral envelope glycoproteins, the F protein and the G protein, are involved in virus entry into the target cell. G is the receptor-binding protein, while F has membrane fusion activity. Both proteins have been shown to elicit VN antibodies [24]. Clearly, antibodies directed to G interfere with binding of RSV virions to cellular receptors, while antibodies directed to F inhibit viral membrane fusion. Therefore, these glycoproteins are promising antigens in the development of a subunit vaccine against RSV [39].

3.1. The F protein

As already mentioned above, the RSV F protein is crucial for virus cell entry, mediating fusion of the viral envelope with the host cell plasma membrane [24]. Through this fusion event, the viral genome is deposited into the cytosol of the host cell. The RSV F protein belongs to the so-called type I viral membrane fusion proteins [24,40]. When the F protein is expressed at the surface of infected cells, it can also induce the formation of syncytia by mediating fusion between neighboring cells [24,40]. The F gene encodes an inactive precursor protein, F_0. A trimer is assembled consisting of three identical F_0 monomers [24]. The

F_0 precursor protein is activated by proteolytic cleavage at two sites [24,41], resulting in the formation of an F_1 (48 kDa) and an F_2 (23 kDa) subunit [42] and the release of the intervening p27 peptide [24,41]. The F_1 and F_2 subunits are covalently linked by two disulfide bonds [24]. The first cleavage occurs in the producer cells and generates the F_2 and the F_1 subunits [43]. There are indications that the second proteolytic cleavage, resulting in the release of p27, occurs after macropinocytosis [43]. It is known that nucleolin on the cell surface can mediate macropinocytosis [44]. Interestingly, Tayyari et al. [45] identified nucleolin as cellular receptor for the F protein, suggesting the involvement of nucleolin in macropinocytosis of RSV. The release of p27 generates a hydrophobic N terminus of F_1, which is known as the fusion peptide of the mature pre-F form of the protein [41,43]. It is the mature pre-F conformation of the F protein that has the capacity to induce membrane fusion by partially refolding and inserting its hydrophobic fusion peptide into the target membrane [24,40,41]. The fusion event is then further facilitated by a conformational change from pre-F to post-F [41]. The trigger for the fusion event is still unknown [24,41]. Figure 3 shows the structures of pre-F and post-F [41].

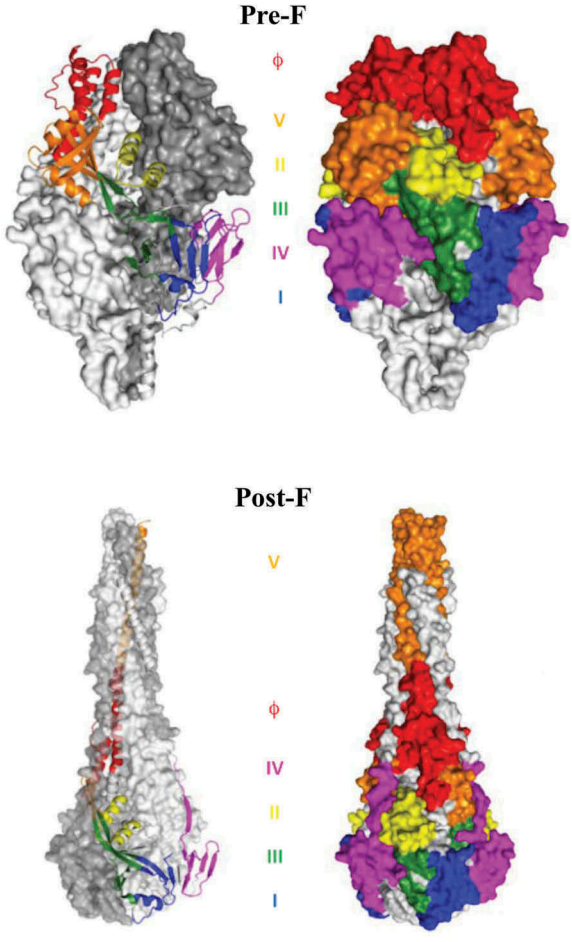

Figure 3. Structures of pre-F (upper) and post-F (lower). The left panels show the structures of pre-F and post-F with one monomer depicted as a ribbon. The right panels show the structures of pre-F and post-F with three monomers. The antigenic sites are indicated with color (red: site Ø, blue: site I, yellow: site II, green: site III, magenta: site IV, and orange: site V). Reproduced with permission from [41].

The F proteins of the two subtypes, RSV A and RSV B, are at least 90% identical in amino acid sequence [24]. As described above, the F protein is located on the surface of the virus and is essential for virus cell entry. This makes the F protein an ideal antigen for the development of subunit-based RSV vaccines [24]. However, both the pre-F and the post-F protein have their shortcomings as antigen for an RSV vaccine. Clearly, effective VN antibodies need to interact with the pre-F conformation of the F protein, thus blocking fusion between the viral envelope and the host cell membrane [46]. Accordingly, it would appear that pre-F is the preferred conformation of F in a vaccine candidate. However, pre-F is a metastable protein, which readily flips into the stable post-F conformation, also in absence of membrane interaction of the protein [46,47]. Post-F may be used as a vaccine antigen because it shares antigenic epitopes with pre-F (sites II and IV, see Figure 3) [41,47,48]. Therefore, antibodies elicited against post-F may still be able to neutralize viral membrane fusion activity mediated by pre-F. However, post-F lacks several epitopes of pre-F, including the well characterized antigenic site Ø (see Figure 3) [24,47,49], which tertiary structure changes during the transition from pre-F to post-F [41]. Other pre-F-specific epitopes, such as the antigenic site VIII (comprising parts of sites II, V, and Ø) [41,50] and a quaternary pre-F-specific epitope (positioned halfway between the membrane-proximal region and the apex of the pre-F trimer) [41,51] have recently been characterized. Therefore, post-F, although stable and thus more practical to work with than pre-F, would appear to be the less favorable antigen for the induction of fusion-inhibitory activity, and thus VN antibodies.

3.2. The G protein

The G protein is glycosylated, which is important for attachment to the target cell [29]. The main receptor for the G protein appears to be the CX3CR1 chemokine receptor [24,52], although other cellular proteins have been implicated in receptor binding of RSV as well [24]. RSV G also binds to heparin sulfate, found on the surface of most cells [24]. The main difference between RSV A and RSV B is the amino acid sequence of the G proteins [28,29]. VN antibodies directed against the G proteins of both RSV subtypes might be needed to provide sufficient protection against RSV [28]. However, it has been shown that the addition of the G protein to F protein-based vaccines may enhance VN antibody titers [53–55].

4. RSV subunit vaccine candidates based on the viral envelope glycoproteins

As of late, the scientific community is getting closer to the development of a safe and effective subunit-based RSV vaccine using the viral envelope glycoproteins as antigens. A concern in the development of an RSV vaccine, is that RSV infections, and thus potentially also vaccination, do not induce (long-lasting) protective immunity. In addition, since subunit-based vaccines are composed of non-replicating component-(s) of the virus, they are often poorly immunogenic [56,57]. Another concern is the quality of the immune response. For optimal protection, a balanced Th1/Th2 response is desired for the induction of immunological memory, cellular immunity,

and potent VN antibodies [58]. To meet these requirements, subunit-based vaccines may require adjuvants and/or multiple administrations to boost the immune response [56–58]. Other ways to boost the immune response and to regulate the Th1/Th2 response include the formulation of virus-like particles (VLPs), nanoparticles, and virosomes. The focus of this review is on subunit-based RSV vaccines using the viral envelope glycoproteins, in particular the F protein, as antigens. These candidates include vaccines based on soluble viral envelope glycoprotein, VLPs, nanoparticles, and virosomes.

4.1. Soluble viral envelope glycoprotein vaccines

Recently, protein engineering has gained considerable interest as a tool to develop effective subunit-based vaccines [59]. By using this technique, tailor-made subunit-based vaccines may be produced and the stability and/or immunogenicity of the vaccine may be improved. Another way to increase the immunogenicity and control the Th1/Th2 response of subunit-based vaccines is the use of adjuvants. However, only a few adjuvants are licensed for human use [56]. Therefore, novel adjuvants have recently been developed and evaluated in combination with subunit-based vaccines. This section reviews strategies applied to develop safe and efficient soluble viral envelope glycoprotein vaccines. Vaccine candidates tested in preclinical studies are first discussed, followed by an overview of candidates tested in clinical studies. Table 1 gives an overview of the *in vivo* studies discussed in this review that used soluble viral envelope glycoprotein, in particular the F protein, as vaccine candidates.

4.1.1. Vaccine candidates in preclinical development

McLellan et al. [49] designed a soluble pre-F antigen called DS-Cav1. It was attempted to stabilize this antigen in the trimeric pre-F conformation by introducing disulfide bonds (DS), filling hydrophobic cavities with hydrophobic substitutions (Cav1), and retaining the C-terminal trimerization domain. A two-dose intramuscular immunization of CB6F1/J mice and rhesus macaques with DS-Cav1 adjuvanted with poly I:C (synthetic double-stranded RNA) resulted in high titers of VN antibodies, directed against the antigenic site Ø of the pre-F protein. These VN antibodies are also known as D25-competing antibodies, as D25 is a monoclonal antibody binding to antigenic site Ø. These high titers make DS-Cav1 a promising candidate for an effective RSV vaccine. However, it has been shown that DS-Cav1 (in an aqueous solution) is not stable during prolonged storage (102 days) at 4°C [60]. The conformation of DS-Cav1 changed to one that was distinct from the pre-F and the post-F conformation of the protein [60]. In the changed conformation, the specific antigenic site Ø was lost, which is an important target for VN antibodies as argued above. Therefore, an improved version of DS-Cav1 was designed, which resulted in the second generation DS-Cav1, named DS2 [61]. In DS2, the F subunits (F_1 and F_2) were genetically linked, the fusion peptides were deleted, and an additional disulfide bond was introduced, reducing the movement between the monomers. According to the authors, a major advantage of DS2 is its improved antigenic stability against heat inactivation (for 60 min at 60°C). In addition, DS2 adjuvanted with poly I:C induced an approximately four-fold higher VN activity than DS-Cav1 in RSV-naïve CB6F1/J mice after two intramuscular immunizations. However, the (long-term) storage stability of DS2 was not investigated. The performed experiments only showed an increased antigenic stability upon heat shock. Moreover, no challenge study was performed. Therefore, further research is needed to demonstrate whether DS2 is suitable as a vaccine antigen.

In 2018, Zhang et al. [62] performed additional structure-based design on DS-Cav1 to improve its stability, leading to a properly folded candidate named F111. To produce F111, the furin cleavage sites, the p27 peptide, and part of the fusion peptide were replaced with a twelve amino acid flexible linker. In addition, an inter-trimer disulfide mutation was introduced. Compared to DS-Cav1, F111 showed an improved heat stability and an improved prolonged storage stability at 4°C (no increased 4D7 (post-F-specific antibody) binding up to three months). However, it is not clear whether D25 binding (antigenic site Ø) remains stable over this period. A two-dose intramuscular immunization of BALB/c mice with F111 adjuvanted with aluminum showed no compromise in immunogenicity compared to DS-Cav1.

Krarup et al. [63] designed a highly stable pre-F vaccine candidate, named SC-DM, from structural analysis of the fusion mechanism. SC-DM is a single-chain (SC), with a short linker between F_1 and F_2 subunits, double mutant (DM) with an optimized apex. Using differential scanning fluorometry, SC-DM showed refolding from pre-F to post-F at 52°C. In addition, heated SC-DM was unable to bind to CR9501, a pre-F-specific antibody. However, it was shown that SC-DM remained in its pre-F conformation for at least 50 days at 4°C, in contrast to DS-Cav1. A two-dose intramuscular immunization of BALB/c mice with SC-DM (with or without poly I:C as adjuvant) resulted in significantly higher neutralizing titers than a two-dose intramuscular immunization with post-F. Moreover, immunization with adjuvanted SC-DM resulted in 10-fold higher VN titers than immunization with adjuvanted post-F. Subsequently, a challenge study was performed in cotton rats with this vaccine candidate. Cotton rats were immunized intramuscularly twice with SC-DM (with or without AdjuPhos, an aluminum phosphate gel, as adjuvant) or twice with post-F (with or without AdjuPhos as adjuvant). The results were comparable to the results obtained in mice and showed a significant enhancing effect of AdjuPhos regarding VN titers elicited by SC-DM. Cotton rats immunized with 5 µg adjuvanted SC-DM were completely protected from RSV challenge.

The 'head-only' antigen designed by Boyington et al. [64] is based on the DS-Cav1 antigen. These authors hypothesized that the head region of the F protein, i.e. the membrane-distal half with an intact antigenic site Ø (see Figure 3), might elicit a better antibody response directed against the antigenic site Ø than the complete F protein. Therefore, the membrane-proximal half (stalk region) of the protein was removed and monomers, dimers, and trimers of the truncated protein were generated. Four of the 'head-only' antigens adjuvanted with poly I:C were studied in CB6F1/J mice. The 'head-only' antigen i-447, a trimeric antigen, showed the most promising results. Following two intramuscular administrations to mice, i-447 elicited VN titers comparable to those induced by DS-Cav1. However, higher levels of antibodies directed against antigenic site Ø were found than with DS-Cav1. Compared to

Table 1. An overview of the *in vivo* studies discussed in this review that used soluble viral envelope glycoprotein, in particular the F protein, as vaccine candidates (study period: 2012–2018).

Preclinical or clinical	Vaccine candidate	Route of administration	Dose vaccine	Adjuvant	Species	Immune response	Reference
Preclinical	DS-Cav1	IM	10 µg	50 µg poly I:C	CB6F1/J mice	High neutralizing antibody titers	[49]
	DS-Cav1	IM	50 µg	500 µg poly I:C	Rhesus macaques	High neutralizing antibody titers	[49]
	DS2	IM	10 µg	50 µg poly I:C	CB6F1/J mice	Four-fold higher neutralizing activity than DS-Cav1	[61]
	F111	IM	2, 0.4, or 0.2 µg	Aluminum adjuvant	BALB/mice	No compromise in immunogenicity compared to DS-Cav1	[62]
	SC-DM	IM	0.5 µg	±50 µg poly I:C	BALB/c mice	Significantly higher neutralizing titers than with post-F	[63]
	SC-DM	IM	0.5 or 5 µg	± AdjuPhos	Cotton rats	Higher neutralization titers than with post-F, adjuvant enhanced neutralizing titers, 5 µg SC-DM + AdjuPhos protected against RSV challenge	[63]
	i-447	IM	10 µg	50 µg poly I:C	CB6F1/J mice	Neutralizing titers comparable to DS-Cav1, higher levels of Ø-directed antibodies than DS-Cav1	[64]
	ΔF/TriAdj	IN	1 µg	10 µg poly I:C + 20 µg IDR1002 + 10 µg PCEP	BALB/c mice	High production IgG1, IgG2a, IgA, B-, Th-, plasma cells, IL-6, IL-21, and TGF-β, longer lasting immunity	[65–67]
	ΔF/TriAdj	IM	50 µg	250 µg poly I:C + 500 µg IDR1002 + 250 µg PCEP	Pregnant ewes	Offspring showed higher IgG, neutralizing antibody titers, lower virus replication, and lower lung pathology than control	[68]
	RSV rF-ptn	IN	2.5–10 µg	W_{80}5EC NE	BALB/c mice	Good IgG and IgA response, high neutralization activity, and enhanced viral clearance	[69]
	F protein	IM	0.3 µg	±100 µg $Al(OH)_3$	BALB/c mice	Higher neutralizing antibodies in young mice than in aged mice (completely protected), adjuvant enhanced immune response in both groups	[70]
	FG-Gb1	IN	50 µg	N/A	BALB/c mice	Higher mucosal and systemic immune response compared to FG alone, mice protected from RSV challenge	[71]
Clinical	RSV-PreF	IM	10, 30, or 60 µg	±500 µg $Al(OH)_3$	Healthy men (18–44 years)	Highest antibody titers 30 µg pre-F/$Al(OH)_3$, 60 µg pre-F/$Al(OH)_3$, and 60 µg pre-F/$Al(OH)_3$, titers decrease at day 60	[72]
	sF	IM	20, 50, or 80 µg	±2.5 µg GLA in 2% SE	Adults (≥60 years)	Dose-dependent increase in humoral and cellular response (neutralizing antibodies, anti-F IgG, and IFN-γ), adjuvant enhanced immune response	[74]
	sF	IM	80 or 120 µg	1, 2.5, or 5 µg GLA in 2% SE	Adults (≥60 years)	Highest immunogenic reaction for 120 µg + 5 µg GLA-SE, acceptable safety profile	[75]
	sF	IM	120 µg	5 µg in 2% SE	Adults (≥60 years)	Vaccine was immunogenic, but did not protect from RSV illness	[76]
	DS-Cav1	IM	50, 150, or 500 µg	±500 µg $Al(OH)_3$	Healthy adults (18–50 years)	Not available yet, estimated study completion date: 3 January 2020	[77]

AdjuPhos: Aluminum phosphate gel; $Al(OH)_3$: Aluminum hydroxide; GLA: Glucopyranosyl lipid A; IDR: Immune defense regulator; IFN-γ: Interferon-γ; IL-6: Interleukin-6; IL-21: Interleukin-21; IM: Intramuscular; IN; Intranasal; N/A: Not applicable; NE: Nanoemulsion; PCEP: Poly[di(sodium carboxylatoethylphenoxy)phosphazene]; Poly I:C: Polyinosinic:polycytidylic acid; SE: Stable emulsion; Transforming growth factor-β; W_{80}5EC: Tween 80 (5%), ethanol (8%), cetyl pyridinium chloride (1%), soybean oil (64%), and water. ± indicates with or without adjuvant.

DS-Cav1, i-447 showed an increased thermostability (no loss of site Ø antigenicity when heated for 60 min at 70°C), although a heat shock for 60 min at 90°C resulted in a substantial loss of site Ø antigenicity (only 10% remaining). Unfortunately, the stability of i-447 upon (prolonged) storage was not investigated. Moreover, a challenge study was not conducted.

Garg et al. [65] showed that a ΔF/TriAdj-vaccine protected rodents for at least one year from RSV infection. The ΔF/TriAdj-vaccine consists of a truncated shortened form of the native F protein (ΔF). To produce ΔF, the transmembrane domain of the protein was deleted. The ΔF protein was formulated with three different adjuvants, namely poly I:C, an immune defense regulator peptide (IDR1002), and a polyphosphazene (poly[di (sodium carboxylate ethyl phenoxy)phosphazene] (PCEP)). Intranasal immunization of BALB/c mice with this vaccine candidate induced higher mucosal IgA production than did two intranasally administered doses of live RSV A2 strain. It also induced higher numbers of B-, Th-, and antibody-secreting plasma cells (in the lymph nodes), and elicited increased expression of interleukin (IL)-6, IL-21, and transforming growth factor-β, resulting in longer lasting immunogenic protection than that induced by infection with live RSV [66]. In 2017, Garg et al. [67] showed that a single dose of intranasally administered ΔF/TriAdj-vaccine also resulted in a long-term protective immunity. BALB/c mice challenged 25 weeks post-immunization showed complete protection against RSV. In another study, ΔF/TriAdj was evaluated as a maternal vaccine [68]. By vaccinating pregnant ewes twice, it was shown that the maternal antibodies (IgG) were transferred to the lambs. The lambs were challenged intranasally with RSV and had lower virus replication and lung pathology than control animals. This indicates that maternal immunization with ΔF/TriAdj might be safe and effective.

Passmore et al. [69] used an intranasally administered recombinant F protein (rF-ptn) adjuvanted with a nanoemulsion. Unfortunately, the conformation of the F protein was not described. The nanoemulsion consisted of Tween 80 (5%), ethanol (8%), cetyl pyridinium chloride (1%), soybean oil (64%), and water. This oil-in-water system with a droplet size of approximately 500 nm was mixed with rF-ptn to obtain the final formulation. The formulation was administered twice, intranasally, to BALB/c mice and gave good humoral (IgG and IgA) responses. High neutralization activity was found and an enhanced viral clearance after RSV challenge was seen. However, as mice were challenged two weeks after administration of the second dose of the vaccine, the duration of the immune response remained unclear.

Cherukuri et al. [70] investigated the effect of the adjuvant $Al(OH)_3$ by intramuscularly vaccinating young (8 weeks) and aged (18 months) BALB/c mice twice with the F protein (conformation not described), with or without the $Al(OH)_3$ adjuvant. After challenge, it appeared that the vaccine with $Al(OH)_3$ protected both young and aged mice to a higher degree than the vaccine without adjuvant. Also, the vaccine with $Al(OH)_3$ induced higher VN antibody titers in young mice (completely protected) than in aged mice. Therefore, a higher antigen dose and/or adjuvant might be needed for the elderly. The duration of the immune response was not investigated, as mice were challenged already two weeks after receiving the second dose of the vaccine.

In 2018, Khan et al. [71] produced FG-Gb1, a recombinant protein meant for intranasal vaccination. FG-Gb1 was produced by genetically fusing the core fragments of the F and the G proteins with Gb-1 (a microfold cell-specific ligand) by using a flexible linker. The authors hypothesized that by using Gb-1, the antigen is targeted to differentiated microfold cells of the nasopharynx-associated lymphoid tissue (NALT), leading to an efficient antigen delivery to the underlying immune induction site. To investigate this, BALB/c mice were intranasally immunized twice with either FG-Gb1 or FG (the fragments without Gb1). Mice that were immunized with FG-Gb1 showed significant increases in antigen-specific serum IgG, IgG-secreting cells in the splenocytes, antigen-specific IgA-secreting cells, sIgA in NALT, and VN antibody titers compared to mice immunized with FG. In addition, mice immunized with FG-Gb1 were protected from RSV challenge. Unfortunately, the stability of the formulation and the duration of the immune response were not investigated.

4.1.2. Vaccine candidates in clinical development

In a phase I study, an RSV vaccine ultimately intended for pregnant women was tested with purified recombinant F protein in the pre-F conformation [72]. It was unclear how the antigen was stabilized in its pre-F conformation. The safety, reactogenicity, and immunogenicity were evaluated. In addition, the use of the adjuvant $Al(OH)_3$ was investigated. In the trial, 128 healthy men, 18–44 years old (of whom 121 completed the study), received an intramuscular injection of the vaccine, containing 10, 30, or 60 µg of pre-F protein, with or without 500 µg $Al(OH)_3$. The vaccine elicited a rapid VN antibody (IgG) response (7 days after vaccination) and had an acceptable adverse events profile. The most commonly reported adverse events were temporary pain at the injection site and mild fatigue. The highest antibody titers were found in the groups that received 30 µg pre-F protein/$Al(OH)_3$, 60 µg pre-F protein/$Al(OH)_3$, or 60 µg pre-F protein without adjuvant. The antibody titers decreased at day 60 and decreased even further at day 180 and day 360. However, VN antibody titers for these three groups at day 360 were still higher than baseline. Unfortunately, no conclusions about the effects of the adjuvant $Al(OH)_3$ could be drawn due to the relatively small sample size. In 2017, a planned phase II study aimed to evaluate the safety, reactogenicity, and immunogenicity of this vaccine in healthy pregnant women and postpartum in their infants was canceled due to instability of the pre-F antigen during manufacturing [73].

Another phase Ia study was conducted with a vaccine containing soluble fusion protein (sF) in adults over 60 years of age [74]. The post-F conformation of the F protein was used. Because the cell-mediated immune system of the target group (the elderly) is in general not easily stimulated, the addition of an adjuvant was applied to boost cellular and humoral immune responses. The synthetic analog of monophosphoryl lipid A, glucopyranosyl lipid A (GLA), was used as an adjuvant, which is a toll-like receptor 4 (TLR4) agonist. GLA was formulated in a 2% squalene-based oil-in-water emulsion (SE). In this study, subjects received the sF vaccine containing 20, 50, or 80 µg post-F protein with or without the adjuvant (2.5 µg of GLA in 2% SE) intramuscularly. The results of the study were

promising, as the safety profile was acceptable for all doses studied and the adjuvant GLA-SE stimulated both humoral and cellular immune responses (micro-neutralization, RSV-specific IgG, and IFN-γ). The immune responses were dose-dependent and did not reach a plateau. Therefore, the efficacy of the vaccine was examined in a larger clinical Ib trial [75]. Subjects of ≥ 60 years received 120 µg sF with different doses of GLA-SE (1, 2.5, or 5 µg), or 80 µg sF with 2.5 µg GLA-SE. The main objectives of the study were to investigate the tolerability and safety of the formulations, and to gather immunogenicity data for dose selection for a subsequent phase II study. The results of the phase Ib study showed that a higher GLA-SE dose augmented local tenderness and pain. However, even for the formulation with the highest dose of GLA-SE, the safety profile remained acceptable. The highest immunogenic response was seen for the formulation containing 120 µg sF adjuvanted with 5 µg GLA-SE. Therefore, this formulation was selected for phase II evaluation. In 2017, the results of this phase II study performed in 1,900 participants of ≥60 years showed that the vaccine was immunogenic, but did not protect from RSV illness [76]. A possible explanation for this outcome, given by the authors, was that the vaccine in the post-F conformation was not able to appropriately generate VN antibodies to prevent RSV illness due to the absence of antigenic site Ø.

In 2017, a phase I study was started with DS-Cav1, despite the fact that this RSV vaccine candidate underwent a conformational change upon long-term storage (102 days) at 4°C. The results of this study are expected to be published soon [77].

4.2. Particulate subunit vaccines

While, in the previous section, we discussed RSV subunit vaccine candidates based on soluble envelope glycoprotein antigens, this section reviews strategies to develop safe and efficient subunit vaccines based on the use of particulate formulations. Particulate vaccine formulations in general offer specific advantages over soluble protein vaccines, including multimeric antigen presentation and improved uptake and processing by antigen-presenting cells [78]. A further distinction within this category is made between vaccine candidates based on virus-like particles (VLPs), nanoparticles, and virosomes.

4.2.1. Virus-like particles (VLPs)

VLPs are supra-molecular complexes, resembling the structure of native viruses. VLPs are generally produced through expression of one or more viral structural proteins, such as surface glycoproteins derived from enveloped viruses, in prokaryotic or eukaryotic expression systems, and often contain a lipid envelope derived from the production cell system [79,80]. Several expression systems for generation of VLPs have been described, including *E. coli*, yeast, insect, or even plant cells [79,80]. A major advantage of VLPs is that they are known to induce potent B- and T-cell responses [81,82]. Using eukaryotic expression systems, VLPs containing RSV envelope glycoproteins have also been produced. This section is devoted to RSV vaccine candidates based on the use of VLPs containing the viral envelope glycoproteins, in particular the F protein. Table 2 gives an overview of the *in vivo* studies discussed in this review that used VLPs containing the viral envelope glycoproteins, in particular the F protein, as vaccine candidates.

Lee et al. [83] investigated whether immunization with a combination of VLPs containing the F protein and VLPs containing the G protein (VLP F + VLP G) leads to an enhanced vaccination efficacy compared to VLPs containing the F protein (VLP F) or VLPs containing the G protein (VLP G) alone. To investigate this, BALB/c mice were vaccinated intramuscularly twice with either VLP F, VLP G, or VLP F + VLP G. All vaccine candidates were in complex with influenza M1, the conformation of the F protein was not investigated. It was shown that mice vaccinated with VLP F + VLP G had lower lung viral loads after RSV challenge, possibly due to an increased CD8 T cell cytokine expression. However, VN antibody titers of mice vaccinated with VLP F alone were similar to mice vaccinated with VLP F + VLP G. Moreover, vaccination with VLP G resulted in an enhanced immunopathology. The duration of the immune response was not investigated, as the challenge experiment was performed three weeks after administration of the second dose of the vaccine.

Table 2. An overview of the *in vivo* studies discussed in this review that used VLPs containing the viral envelope glycoproteins, in particular the F protein, as vaccine candidates (study period: 2012–2018).

Vaccine candidate	Route of administration	Dose vaccine	Adjuvant	Species	Immune response	Reference
VLP F, VLP G, or VLP F + VLP G	IM	25 µg (12.5 µg + 12.5 µg for VLP F + VLP G)	N/A	BALB/c mice	VLP F + VLP G showed lower lung viral loads and higher $CD8^+$ T cell cytokine expression	[83]
rNDV/RSV/F, rNDV/RSV/G, or rNDV/RSV/F + G	IN	40 µg (20 µg + 20 µg for rNDV/RSV/F + G)	N/A	BALB/c mice	rNDV/RSV/F + G showed highest serum IgG, highest nasal serum IgA, highest IFN-γ and IL-4, and better protection after challenge	[84]
Post-F VLP/pre-F VLP/combo VLP	IM	4 µg	Squalene-based oil-in-water NE	BALB/c mice	Complete protection for all candidates, combo VLP highest neutralizing potency and a balanced Th1/Th2 response	[85]
VLP-H/G+ pre-F/F or VLP-H/G + post-F/F	IM	30 µg	N/A	BALB/c mice	High VN antibody titers for VLP-H/G+ pre-F/F, low VN antibody titers for VLP-H/G+ post-F/F	[86]
VLP-H/G+ pre-F/F or VLP-H/G + post-F/F	IM	100 µg	N/A	Cotton rats	VLP-H/G+ pre-F/F most efficient in boosting VN antibodies in mothers, pups showed enhanced VN antibody titers and protection of the lung from RSV replication after challenge	[87]

IFN-γ: Interferon-γ; IL-4: Interleukin-4; IM: Intramuscular; IN; Intranasal; N/A: Not applicable. ± indicates with or without adjuvant.

In a comparable study, Newcastle disease virus (NDV) was used as platform for VLPs containing the RSV F protein (rNDV/RSV/F) and/or G protein (rNDV/RSV/G) [84]. In this study, mice were intranasally vaccinated twice with either rNDV/RSV/F, rNDV/RSV/G, or a combination of both (rNDV/RSV/F + G). Mice that were vaccinated with rNDV/RSV/F + G showed the highest serum IgG titers, the highest nasal serum IgA titers, and highest levels of IFN-γ and IL-4. Moreover, mice vaccinated with rNDV/RSV/F + G showed better protection after RSV challenge than mice vaccinated with rNDV/RSV/F or rNDV/RSV/G alone. Unfortunately, the conformation of the F protein was not described. In addition, it is not clear when the challenge experiments were performed.

Cimica et al. [85] used the M protein of human metapneumovirus as scaffold together with recombinant post-F, pre-F, or a combination of both for their VLPs. The pre-F protein was stabilized by introducing two disulfide bonds. A squalene-based oil-in-water emulsion was used as adjuvant. Two intramuscular administrations with the vaccine containing both post-F and pre-F protein yielded complete protection against RSV challenge and no viral lung replication in challenged BALB/c mice was seen. Furthermore, this formulation showed a balanced Th1/Th2 response and gave the highest VN antibody titers, followed by the pre-F only formulation. The post-F protein VLP vaccine gave the lowest neutralizing antibody titers. Unfortunately, the (prolonged) stability of the formulation was not tested. Moreover, the duration of the immune response was not investigated, as the challenge experiment was performed already two weeks after administration of the second dose of the vaccine.

Cullen et al. [86] investigated whether previously RSV-infected mice generated protective immune responses when vaccinated with their VLPs. This study was performed to investigate the effect of pre-existing immunity on vaccination efficacy, which is important for vaccine candidates meant for pregnant women and the elderly. The authors used NDV core proteins to develop VLPs containing chimera H/G protein and either chimera pre-F/F (VLP-H/G+ pre-F/F) or chimera post-F/F (VLP-H/G+ post-F/F), to investigate the influence of the conformation of the F protein on the immune response. Mice were first infected with RSV by intranasal inoculation. Then, 95 days post-infection, mice were immunized intramuscularly with either VLP-H/G+ pre-F/F or VLP-H/G+ post-F/F. After vaccination, high VN antibody titers were observed in mice that were immunized with VLP-H/G+ pre-F/F, in contrast to mice that were immunized with VLP-H/G+ post-F/F. These results indicate that VLPs containing the F protein in its pre-F conformation are able to induce a VN antibody memory response, in contrast to VLPs containing the post-F conformation. Therefore, the conformation of the F protein seems to be of great importance in vaccine candidates intended for pregnant women and the elderly.

In 2018, the efficacy of VLP-H/G+ pre-F/F and VLP-H/G + post-F/F was assessed in a maternal immunization model using cotton rats [87]. A subgroup of female cotton rats was intranasally infected with RSV. Subsequently, all female cotton rats were set up in breeding pairs at week 8. At week 10, pregnant cotton rats were intramuscularly vaccinated with VLP-H/G+ pre-F/F, VLP-H/G+ post-F/F, purified pre-F, or purified post-F. At 12 weeks, cotton rats delivered their pups, which were intranasally challenged with RSV at 4 weeks of age. The results of this study were comparable to the results of the previous study and showed that VLPs containing pre-F were more efficient in boosting pre-existing VN antibodies than VLPs containing post-F. In addition, VLPs containing pre-F were also more efficient than purified F protein (pre-F as well as post-F). Pups born to mothers that were immunized with VLP-H/G+ pre-F/F showed enhanced VN antibody titers and protection of the lung from RSV replication after challenge. Unfortunately, the (prolonged) stability of the formulation was not tested.

4.2.2. Nanoparticles

In recent years, the use of nanotechnology in vaccine development increased significantly. Nanoparticles are more simple structures than VLPs, usually lacking a lipid envelope and consisting of a self-assembled oligomeric viral protein, such as – for example – RSV F. The main advantages of nanoparticle-based vaccines are an increased antigen stability and an enhanced immunogenicity [88,89]. Table 3 gives an overview of the *in vivo* studies discussed in this review that used nanoparticles containing the viral envelope glycoproteins, in particular the F protein, as vaccine candidates.

A recombinant F protein nanoparticle vaccine was developed by Smith et al. [90]. This vaccine contained a modified almost full length F protein produced in *Spodoptera frugiperda* insect cells infected with a recombinant baculovirus. The nanoparticles (approximately 40 nm in size) were composed of F protein oligomers arranged in the form of rosettes without a lipid envelope. The F protein oligomers had a morphology consistent with the post-F conformation. Different doses (1, 6, or 30 μg) with or without 120 μg $AlPO_4$ as adjuvant were tested. All doses protected cotton rats from RSV challenge after a two-dose intramuscular immunization. High serum IgG levels and neutralizing activity were found for the animals vaccinated with the F protein nanoparticles, the highest titers were found with the adjuvanted vaccine candidate. Immunization of cotton rats with the vaccine containing $AlPO_4$ elicited serum levels of palivizumab-competing antibodies greater than after passive administration of palivizumab [91]. Therefore, the vaccine is potentially more broadly protective than palivizumab. Unfortunately, no (prolonged) stability data were available and the duration of the immune response is not clear, as the animals were challenged 4 weeks after receiving the second dose of the vaccine.

Recombinant F protein nanoparticle vaccines based on the vaccine developed by Smith et al. [90] described above, are under clinical evaluation. The vaccines are meant for pregnant women in their third trimester, the elderly, and children with an age between 6 months and 5 years. In a phase III study with 11,850 participants (≥60 years), the nanoparticle vaccine meant for the elderly failed to show efficacy against RSV moderate-severe lower respiratory tract disease [92–94].

As a phase III study for the vaccine meant for pregnant women is still ongoing, this vaccine will be discussed in more detail. The nanoparticle vaccine for pregnant women was first tested in pregnant guinea pigs [95] and two clinical trials in

Table 3. An overview of the *in vivo* studies discussed in this review that used nanoparticles containing the viral envelope glycoproteins, in particular the F protein, as vaccine candidates (study period 2012–2018).

Vaccine candidate	Route of administration	Dose vaccine	Adjuvant	Species	Immune response	Reference
RSV F nanoparticles	IM	1, 6, or 30 µg	±120 µg $AlPO_4$	Cotton rats	High IgG levels, neutralizing activity, protection from challenge for all doses	[90]
RSV F nanoparticles	IM	30 µg	±$AlPO_4$	Cotton rats	Adjuvanted vaccine induced higher serum levels of palivizumab-competing antibodies than passive administration of palivizumab	[91]
RSV F nanoparticles	IM	135 µg	N/A	Adults (≥60 years)	Vaccine did not show efficacy against RSV moderate-severe lower respiratory tract disease	[92–94]
RSV F nanoparticles	IM	30 µg	±400 µg $AlPO_4$	Pregnant guinea pigs	High IgG titers and neutralizing activity, adjuvant enhances immune response	[95]
RSV F nanoparticles	IM	60 or 90 µg	±1.2 mg $AlPO_4$	Healthy women (18–35 years)	High IgG antibody levels, increased microneutralization antibodies, best results with 2 adjuvanted doses	[96]
RSV F nanoparticles	IM	60 or 120 µg	0.2, 0.4, or 0.8 mg $AlPO_4$	Healthy women (18–35 years)	Optimal formulation: one dose 120 µg adjuvanted with 0.4 mg $AlPO_4$	[97]
RSV F nanoparticles	IM	120 µg	0.4 mg $AlPO_4$	Third trimester pregnant women (18–40 years)	The efficacy against RSV lower respiratory tract infections through 90 days of life in infants was 39.4%, trial did not meet its primary objective	[98–100]
RSV F nanoparticles	IM	10 µg	±60 µg AdjuPhos	Cotton rats	Induction of antibodies targeting multiple neutralizing epitopes, use of adjuvant resulted in enhanced protection against RSV challenge	[101]
Coupled RSV F/TRP	SC	3–5 µg	5.15 nmol TLR7/8a	CB6F1/J mice	High pre-F specific antibody titers mediated by Th1, protection against RSV challenge	[102]

AdjuPhos: Aluminum phosphate gel; $AlPO_4$: Aluminum phosphate; IM: Intramuscular; SC: Subcutaneous; TLR7/8a: Toll-like receptor7/8a. ± indicates with or without adjuvant.

healthy women of childbearing age were subsequently performed. The pregnant guinea pigs were immunized twice with 30 µg F protein with or without 400 µg $AlPO_4$. Both the non-adjuvanted and adjuvanted vaccine yielded high IgG titers, palivizumab-competing antibodies, and serum showed RSV neutralizing activity *in vitro*. However, the adjuvanted vaccine gave a more potent immune response. The results showed adequate antibody transfer to the pups for both the adjuvanted and the non-adjuvanted groups. In the first phase II clinical study, healthy non-pregnant women (18–35 years old) were intramuscularly immunized with 1 or 2 dose(s) of the vaccine (60 or 90 µg) with or without 1.2 mg $AlPO_4$ as adjuvant [96]. The vaccine appeared to be safe, immunogenic, and reduced RSV infections. High serum IgG antibody levels were found in all groups, which were still detectable after 112 days. The best results were obtained when the vaccine with adjuvant was administered twice. A modest increase in antibody levels was observed for the higher antigen dose (90 µg). For the second phase II study, non-pregnant women (18–35 years old) were vaccinated intramuscularly with 1 or 2 dose(s) of 60 µg or 120 µg adjuvanted with 0.2, 0.4, or 0.8 mg $AlPO_4$ [97]. All formulations were immunogenic and well tolerated. The formulation of a single dose of 120 µg adjuvanted with 0.4 mg $AlPO_4$ was the formulation with an optimal combination in terms of efficacy and convenience. The safety and immunogenicity of the F protein nanoparticle vaccine, with $AlPO_4$, is currently being studied in healthy, pregnant women in their third trimester. In this phase III study, 4,636 third trimester pregnant women were enrolled [98,99]. The study completion date is estimated by July 2019. However, it was recently reported that the study did not meet its primary objective (prevention of medically significant RSV lower respiratory tract infections) [99]. The efficacy of the vaccine through 90 days of life in infants against medically significant RSV lower respiratory tract infections was reported to be 39.4% [100].

In 2018, Gilbert et al. [101] characterized the immune response to the RSV F nanoparticle vaccine described above. To this end, the authors immunized cotton rats intramuscularly twice with 10 µg of the RSV F nanoparticle vaccine with or without 60 µg aluminum phosphate as adjuvant. Subsequently, sera were analyzed on cross-competition of F antigenic site specific monoclonal antibodies, namely: D25 for site Ø (pre-F-specific), motavizumab for site II, NVX for site IV, and hRSV90 for site VIII (pre-F-specific). Despite the fact that a morphology consistent with that of the post-F conformation was previously reported for this candidate [90], the serum of cotton rats that had been immunized with the RSV F nanoparticle vaccine cross-competed with all monoclonal antibodies, including the pre-F-specific monoclonal antibodies D25 and hRSV90. This was also supported by the fact that cotton rats immunized with the RSV F nanoparticle vaccine were protected against challenge with a palivizumab-resistant mutant virus. These data show that multiple neutralizing epitopes, including the pre-F-specific sites Ø and VIII, are targeted upon immunization with the RSV F nanoparticle vaccine. Cotton rats immunized with the adjuvanted vaccine showed higher anti-F IgG and micro neutralizing titers. In addition, the adjuvant increased site II antibody avidity, which resulted in an enhanced protection against RSV challenge.

Francica et al. [102] used TLR agonists as adjuvants to an F protein (in the pre-F conformation (DS-Cav1)) vaccine to enhance antibody responses in CB6F1/J mice. The authors discovered that conjugation of F protein to TLR-7/8a disrupts the recognition of critical VN epitopes. However, if the

F protein was coupled to nanoparticle-forming thermoresponsive diblock co-polymers (consisting of N-(2-hydroxypropyl) methacrylamide (HPMA) and di(ethylene glycol) methyl ether methacrylate (DEGMA)) and if TLR-7/8a was used as adjuvant, high titers of pre-F specific antibodies mediated by Th1 cells were obtained. This formulation seems to have appropriate antigenicity, as the mice were fully protected from RSV challenge after being immunized subcutaneously twice. However, the duration of the immune response is unclear, since the mice were challenged only 17 days after vaccination. Moreover, the (long-term) storage stability of the formulation was not investigated.

4.2.3. Virosomes

Virosomes are VLPs, in the sense that they closely resemble the virus they are derived from. However, unlike the VLPs discussed above in section 5.2.1, virosomes are produced from native virus through a detergent solubilization and subsequent reconstitution procedure. Thus, virosomes are reconstituted viral envelopes. They lack the viral genetic material and therefore are non-replicating. Virosomes have been originally produced from influenza virus [103,104]. Using the detergent octaethyleneglycol mono (n-dodecyl) ether ($C_{12}E_8$), Stegmann et al. [104] demonstrated that the envelope of influenza virions could be efficiently solubilized; after sedimentation of the viral nucleocapsid through ultracentrifugation and subsequent removal of the $C_{12}E_8$ from the supernatant by the addition of a hydrophobic resin, reconstituted viral envelopes were formed which retained the viral receptor-binding and membrane fusion activities. In a follow-up study, the virosomal production procedure was adjusted; $C_{12}E_8$ was replaced by a short-chain phospholipid, dicaproylphosphatidylcholine (DCPC) [105]. Unlike $C_{12}E_8$, DCPC is removable by dialysis because of its high critical micelle concentration.

Since virosomes retain the morphology and cell interaction characteristics of the native virus, they represent excellent non-replicating vaccine formulations. This has been demonstrated extensively for virosomes derived from influenza virus [106]. Not only can influenza virosomes be used as vaccine against influenza infection, they may also be employed as carriers of foreign antigens, for induction of both antibody and cell-mediated immune responses, including CD8 T cell responses [106]. An additional advantage of virosomes relates to the opportunity to incorporate lipophilic or amphiphilic adjuvants in the virosome membrane during the reconstitution procedure [106]. Table 4 shows an overview of the *in vivo* studies discussed in this review that used RSV virosomes as vaccine candidates.

Using the DCPC-dialysis procedure, virosomes have been produced from RSV and evaluated for their immunogenicity in animal model systems. Initial studies were carried out by Stegmann et al. [107] and Kamphuis et al. [108–110], who incorporated TLR2- or TLR4-ligands as vaccine adjuvants in the virosomal membrane, respectively. When administered intramuscularly to mice or cotton rats, these virosomes induced robust VN antibody and T helper cell responses, with a balanced Th1/Th2 signature. Also, they provided complete protection from viral infection upon challenge [107,108,110]. In addition, in mice [109,111] and cotton rats [109], protective immunity may also be induced by intranasal administration of adjuvanted virosomal RSV vaccines.

More recently, the incorporation of TLR4-ligands, in particular non-toxic variants of bacterial monophosphoryl lipid A (MPLA), as adjuvants in virosomal RSV candidates has further been investigated [112]. An important improvement to the use of this potent adjuvant system relates to the use of the fully synthetic MPLA-derivative 3D-PHAD®, produced by Avanti Polar Lipids, allowing vaccine production under GMP conditions. The authors of the latter study also investigated the morphology and long-term stability of the 3D-PHAD-containing RSV virosomes. Single particle tracking showed that the virosomes were stable for 300 days at 4°C.

5. Conclusion

For a long time, there has been a reluctance towards the development of an RSV vaccine due to the failure and dramatic outcome of a study published in 1969, in which two young children, vaccinated with an experimental FI-RSV vaccine, died due to vaccine-enhanced respiratory disease after exposure to a natural RSV infection [17]. Since this disastrous FI-RSV vaccine trial, vaccination of very young children against RSV has remained elusive. However, there is now a growing interest in vaccination of pregnant women to passively protect infants against RSV in the crucial first months of their life. Subunit-based vaccines are explicitly considered for this purpose. In addition, this type of vaccine is also considered for

Table 4. An overview of the *in vivo* studies discussed in this review that used RSV virosomes as vaccine candidates (study period: 2012–2018).

Vaccine candidate	Route of administration	Dose vaccine	Adjuvant	Species	Immune response	Reference
RSV virosomes	IM	5 µg	± P3CSK4	BALB/c mice and cotton rats	Robust virus-neutralizing antibody and T helper cell responses, balanced Th1/Th2, complete protection upon challenge	[107]
RSV virosomes	IM	5 µg	± MPLA	BALB/c mice and cotton rats	Robust virus-neutralizing antibody and T helper cell responses, balanced Th/Th2, complete protection upon challenge	[108,110]
RSV virosomes	IN	5 µg	± MPLA	BALB/c mice and cotton rats	Induction of protective immunity	[109]
RSV virosomes	IN	5 µg	± P3CSK4 and/or L18-MDP	BALB/c mice	Induction of protective immunity	[111]

IM: Intramuscular; IN; Intranasal; L18-MDP: 6-O-stearoyl-N-Acetyl-muramyl-L-anayl-D-isoglutamine; MPLA: Monophosphoryl lipid A; P3CSK4: N-palmitoyl-S-[2,3-bis(palmitoyloxy)-(2RS)-propyl]-[R]-cysteinyl-[S]-seryl-[S]-$(lysyl)_3$-lysine. ± indicates with or without adjuvant.

vaccinating another important target population, the elderly. This is the reason why over the last years, research has been intensified toward development of safe and effective subunit-based RSV vaccines. This review focused on subunit-based RSV vaccines using the viral envelope glycoproteins, in particular the F protein, as antigens. The F protein has proven to be an effective antigen in both the pre-F and post-F conformation, and subunit-based vaccine candidates using the F protein as antigen have been shown to elicit high titers of VN antibodies. However, as yet, no vaccine candidate has been able to elicit robust protective immunity, as demonstrated by multiple failed clinical trials. Three of the recently failed clinical studies used vaccine candidates with post-F protein or with a morphology consistent with that of the post-F conformation [76,92–94,98–100], suggesting the importance of the pre-F-specific antigenic sites and the pre-F morphology in eliciting protective immunity. Although a morphology consistent with that of the post-F conformation was reported for the nanoparticle-based vaccine candidate [90], it has been shown that the vaccine candidate induced antibodies that competed with pre-F-specific monoclonal antibodies [101], suggesting unknown factors in RSV subunit vaccine development. The of use of pre-F as antigen presents stability challenges, as demonstrated by a recently canceled phase II study with a vaccine candidate in the pre-F conformation [73]. Therefore, induction of protective immunity and preservation of vaccine stability remain challenges in the development of subunit-based RSV vaccines using the viral envelope glycoproteins as antigens.

6. Expert opinion

Although many of the vaccine candidates described in this review showed promising results in animal models and some even in human trials, apparent weaknesses are obvious and should be addressed as well. Most studies showed that immunization with the vaccine candidate resulted in high titers of neutralizing antibodies. However, *in vivo* challenge experiments were not always carried out. The ultimate goal of vaccination is to elicit (long-lasting) protective immunity. While some animal studies did include *in vivo* challenge experiments, these were usually carried out already 2–5 weeks after the (booster) immunization. This is rather a short period of time, since it is well known that humoral immunity against RSV decreases over time [72,113]. By contrast, there are some studies that have investigated the induction of long-term memory in animals [65–67]. These studies showed that it was possible to induce long-term memory with a subunit RSV vaccine based on the F protein. However, that eliciting a protective immune response is far from trivial is also indicated by the failure of a phase II [76] and two phase III [92–94,98–100] studies. For these trials, the post-F protein or a nanoparticle-based vaccine candidate with a morphology consistent with that of the post-F conformation was used, respectively. The post-F conformation, as previously discussed, lacks important pre-F-specific antigenic sites, such as antigenic sites Ø and VIII [47,49,50]. The absence of these important epitopes may be the reason why the phase II trial failed. However, with the nanoparticle-based vaccine candidate, which had a morphology consistent with that of post-F [90], antibodies competing with pre-F-specific monoclonal antibodies were induced [101]. These results suggest unknown factors in RSV subunit vaccine development.

A vaccine candidate based on pre-F might be more immunogenic. However, using pre-F as antigen presents its own challenges. In 2017, a phase II study with a vaccine containing pre-F was canceled due to instability of the pre-F antigen during manufacturing [73]. This illustrates the major concern regarding the use of pre-F as antigen. Modified vaccines based on the pre-F antigen were developed. However, except for two formulations (DS-Cav1 and SC-DM), no data are available on the storage stability of these vaccine candidates. It should furthermore be noticed that all formulations described in this review are liquid (aqueous) formulations, with concomitant disadvantages, such as a poor (storage) stability. To prevent deterioration, liquid formulations require strictly monitored refrigeration during storage and transport, known as the 'cold-chain', which implicates logistic challenges. To overcome the disadvantages related to liquid formulations, the development of a dry powder vaccine may be an elegant solution [114–116]. However, an important consideration to take into account is that the drying process itself may be detrimental to the antigen, i.e. it may result in conformational changes and/or loss of activity [114]. Excipients can be used to prevent this. Certain sugars (e.g. trehalose, sucrose, or inulin) are known to stabilize proteins during drying and subsequent storage and are therefore interesting excipients to be used for the development of such dry powder vaccines [116,117]. Although to our knowledge, pre-F has never been stabilized by drying, several other subunit vaccines have been processed into a stable dry powder, e.g. influenza haemagglutinin [118] and hepatitis B surface antigen [119].

A dry powder RSV vaccine based on pre-F would not only show improved (storage) stability, it might also increase the efficacy of intranasal administration of the vaccine. A disadvantage of liquid intranasal vaccine formulations is their generally poor absorption over the nasal mucosa, limiting their efficacy. This problem can be overcome by incorporating mucoadhesive excipients, such as chitosan, in a dry powder vaccine formulation [120]. This will improve the absorption of the vaccine over the nasal mucosa and therefore increase the efficacy of the vaccine formulation, but may also have adverse effects, including ciliotoxicity, that should be considered when alternative formulations are developed.

A major advantage of a dry powder RSV vaccine based on pre-F would be the possibility of pulmonary administration. This route of administration is interesting because pulmonary vaccination against other airborne diseases (influenza and measles) has been proven successful in humans in several studies [121–126]. Furthermore, the lung is the site where RSV-related pneumonia takes place [127]. The pulmonary administration route for vaccines has the potential to elicit both a systemic (IgG) and mucosal (IgA) immune response [114,115]. It is known that RSV-specific IgA has a protective role against RSV infection [128]. This could lead to an enhanced (longer-lasting and stronger) immune response [115]. Indeed, Amorij et al. [129], using an influenza subunit vaccine, have demonstrated that the pulmonary administration route has

the capacity to elicit robust systemic humoral (IgG), mucosal humoral (IgG and IgA), and cell-mediated immune responses in BALB/c mice. Pulmonary administered dry powder measles vaccine formulations have also been successfully used in cotton rats [130] and rhesus macaques [131]. A pulmonary administered dry powder vaccine has several advantages over a pulmonary administered liquid vaccine, as described by Tonnis et al. [132]. However, for such a pulmonary vaccine to be successful, a combination between a suitable delivery device and an optimal vaccine formulation is required [132]. For pregnant women and for the elderly, a dry powder vaccine could be administered by using a disposable (single-dose) inhaler such as the Twincer®. The ability of this inhaler to accurately deliver drugs and its ease in use have been documented [133]. In conclusion, the development of a dry powder subunit-based RSV vaccine formulation using pre-F for pulmonary administration may overcome the disadvantages presented by most vaccine candidates discussed in this review.

In the near future, the development of subunit-based RSV vaccines using the viral envelope glycoproteins, in particular the F protein, as antigens will remain an interesting approach in the development of effective RSV vaccines. The failure of three recent clinical trials will shift research into the direction of antigens in the pre-F conformation and with a morphology consistent with that of pre-F, since this form has important pre-F-specific antigenic sites, including antigenic sites Ø and VIII. In view of the poor stability of this conformation, innovative stabilization technologies are needed and they will be essential in developing vaccine concepts that are suitable for widespread use. Finally, research toward the development of dry powder subunit-based RSV vaccines, based on stabilized pre-F, will be worthwhile. Pulmonary administration of such dry powder RSV vaccines should be explored as it may yield clinically relevant results.

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